THE IN VITRO REACTION OF LIMULUS AMEBOCYTES TO BACTERIA [1]

M. V. SHIRODKAR,[2] A. WARWICK AND F. B. BANG

Marine Biological Laboratory, Woods Hole, Massachusetts, and Department of Pathobiology, The Johns Hopkins University School of Hygiene and Public Health, Baltimore 5, Maryland

The horseshoe crab, *Limulus polyphemus,* an ancient marine arachnoid, reacts to infection by extensive intravascular clotting (Bang, 1956). Since this is primarily a cellular reaction, followed by extracellular gelation (Loeb, 1910), it was of particular interest to study the interaction of bacteria and the cellular components *in vitro.* The present paper reports (a) the maintenance of the normal granular discoid amebocytes in siliconized and unsiliconized glassware, (b) the destructive effect of the bacteria and bacterial toxin in first changing these cells to agranular and filiform extended cells and then destroying them, and (c) the ability of the granular discoid amebocytic preparations to suppress the development of nonpathogenic bacterial infections in the cultures.[3]

Materials and Methods

Healthy, adult specimens of *Limulus,* 10 to 12 inches across the shell, mostly female, were obtained from the Marine Biological Laboratory Supply Department and kept in running sea water. No data were available concerning the previous history of infection among these animals.

Preparations without silicone—granular and agranular cells

Thirty to 50 ml. of blood were obtained by cardiac puncture and were placed directly into a series of unsiliconized 10-ml. screw-cap roller tissue culture tubes (2 ml. per tube) with routine sterile precautions. After some manipulations to obtain a uniformly thin layer of material over most of its inner curvature, the tube was placed in a rotating drum and kept at room temperature (20–25° C.). The cells were studied by direct microscopic examiantion of the tube and by removal of drops of the culture fluid [4] with its suspended amebocytes. The latter were followed both by phase and direct transmitted light. A combination of 100 units each of penicillin and streptomycin was used in a few of the early experiments, but in most tubes no antibiotics were used. The effect of fluid changes was studied by replacing the media with various concentrations of adult

[1] This work was supported by a Grant-in-Aid (E 135) from the National Institutes of Health.

[2] Predoctoral Research Fellow, National Institutes of Health.

[3] Some of this material has been reported in abstracts: (Shirodkar, Bang and Warwick, 1958; Warwick and Bang, 1957).

[4] Hereafter referred to as "medium" or as "serum."

Limulus serum. Wolbach's modification of Giemsa's stain was used in order to determine the cytological details of the cells.

Cultures with silicone—cultures in siliconized glassware

In these experiments the culture tubes, syringes and needles were coated with silicone (G.E. SC-87 Dri-Film) and air-dried for 3–4 hours before they were thoroughly washed with the commercial washing powder "Gold Dust," rinsed several times with tap and then with distilled water and dry sterilized at 180° C. for 1½ hours. Large volumes of blood could not be withdrawn and transferred (2 ml. for each roller tube) without clot formation unless the apparatus was thus siliconized. Particularly careful manipulation was required immediately after explantation of the blood. The preparation was considered satisfactory only if a clear majority of the attached and suspended amebocytes was indistinguishable from those found *in vivo, i.e.* discoid, granular, (Lankester, 1884; Loeb, 1902), as studied by low power microscopy, for at least 48 hours following explantation.

Description of bacteria used

Limulus pathogen. In the summers of 1953, 1954, 1957 and 1958 organisms were isolated from the peripheral blood of sick adult animals by means of techniques described elsewhere, and the 1958 stock cultures were maintained by fortnightly transfers on ZoBell's sea water agar, enriched with peptone and ferric salts (Bang, 1956). They were stored between transfers at 4° C. On the basis of bacteriological tests [5] the strain isolated in 1958 was identified as a *Vibrio* sp. It is gram-negative, short, thick, polarly monotrichous, motile and does not form spores. It is comma-shaped, particularly in old cultures. Thin capsules are occasionally present. The organism is facultatively anaerobic and has an optimum growth temperature of 22° C., complete cessation of growth occurring at 5° and 37° C. It produces β-hemolytic zones on horse blood agar; liquefies gelatin rapidly (1 day at 22° C.); shows presence of peroxidase and catalase; does not produce H_2S; gives a weak positive reaction with indole and reduces nitrate to nitrite. The bacterium is incapable of fixing nitrogen and shows neither fluorescence nor luminescence. It produces acid, but not gas, in the following carbohydrate media: glucose, galactose, maltose, trehalose, mannitol, starch, sorbitol (weak at 15 days), glycerol (weak) and salicin (weak). No acid is produced with arabinose (late positive, 15 days), xylose, rhamnose, sucrose, lactose, adonitol, dulcitol, inositol and ethyl alcohol.

A heat-stable toxin was obtained from this bacterium by the same method as in the previous work.

Bacterium #5 (Limulus nonpathogen). Pure cultures [6] of this gram-negative peritrichous, motile rod were obtained at the beginning of the summer of 1958 from oysters.

Bacteriological studies showed no fermentation except in ethyl alcohol, which

[5] These, as well as the tests on Bacterium #5, were kindly performed by Dr. H. Lautrop of the State Serum Institute, Copenhagen, Denmark, to whom we are grateful for the accompanying description.

[6] These, as well as other cultures used here, were isolated by Mr. Stuart Krassner of our laboratory and kindly supplied to us.

suggested that it belongs in the Alkaligenes group. However, since it produces H_2S, it is tentatively designated Alkaligenes-like. When inoculated, even in large doses, into healthy, adult animals, these bacteria produced no demonstrable disease.

Other bacteria. Eight different bacteria were tested, seven of which had been isolated from oysters whilst one, a yellow chromogen (#19), was found as a laboratory contaminant. No attempt was made to fully identify any of them. They were distinguished one from the other principally on the basis of colony and cellular morphology. They were arbitrarily numbered 4, 10, 12, 14, 15, 17, 18, 19.

Antibacterial activity of cultures. The experiments designed to test the extent of antibacterial activity and to determine in which fraction(s) of blood it resided, consisted essentially of (a) setting up blood culture tubes, adding known amounts of bacteria, withdrawing aliquots from the mixtures at successive time intervals and plating these on agar to determine the drop in numbers of viable bacteria; (b) following the same procedure with tubes containing different fractions of the blood, such as serum with few granules, serum with many granules and with fresh, whole blood homogenates. The measures of antibacterial activity were (1) the largest initial inoculum following whose introduction into the system either no bacteria (or reduced numbers) were recoverable and (2) the length of time needed to achieve such reduction or to maintain inhibition at a constant level.

One ml. of sterile, artificial sea water (without $NaHCO_3$) was added to an agar slant containing a heavy 24–48 hour (room temperature) growth of the organism and mixed thoroughly. In the earlier experiments, the titers of such undiluted suspensions of the various bacteria were determined by making pour plates of serial $\log_{10}$ dilutions in sea water agar. The resulting values for viable bacteria were found to be consistently in the range of 10^9 to 10^{10} cells per 0.1 ml. We subsequently assumed these figures for freshly prepared suspensions. Inocula of 0.1 ml. of various dilutions were added to the experimental and control culture tubes; the tubes were kept at room temperature in the rotating drum, and samples were taken subsequently for culture by means of a platinum wire loop. Colonies were counted at 24 hours. In the series of experiments designed to yield information regarding the fraction(s) of blood containing the factor(s) responsible for the bacteria-reducing capacity of the cultures, fresh blood was aseptically explanted into unsiliconized Petri dishes which were then allowed to stand at room temperature for varying periods of time. A gel-like clot formed rapidly after explantation and was adherent to the bottom of the dish as described by Loeb (1920). Syneresis occurred and the supernatant serum contained a minimum number of free amebocyte cytoplasmic granules if drawn off within a few minutes after placing the blood in the dish. With stirring, this number increased steadily up to about two hours. Aliquots were removed from the supernate at intervals and the state of the granules was noted under the low and high dry powers of the light microscope, using blue light and no filter, with reference to shape, size, color, refractility and Brownian movement.

Three terms which will have particular connotations as used throughout this report may need clarification. A *normal granule* is one which appears like those seen in the intact cell. It has a uniform size; the shape is spherical to slightly ovoid; it appears green when seen through the ordinary light microscope, using blue light and no filter; has high refractility and shows a characteristic amplitude of Brownian movement when in serum. *Polymorphic granules* are considered

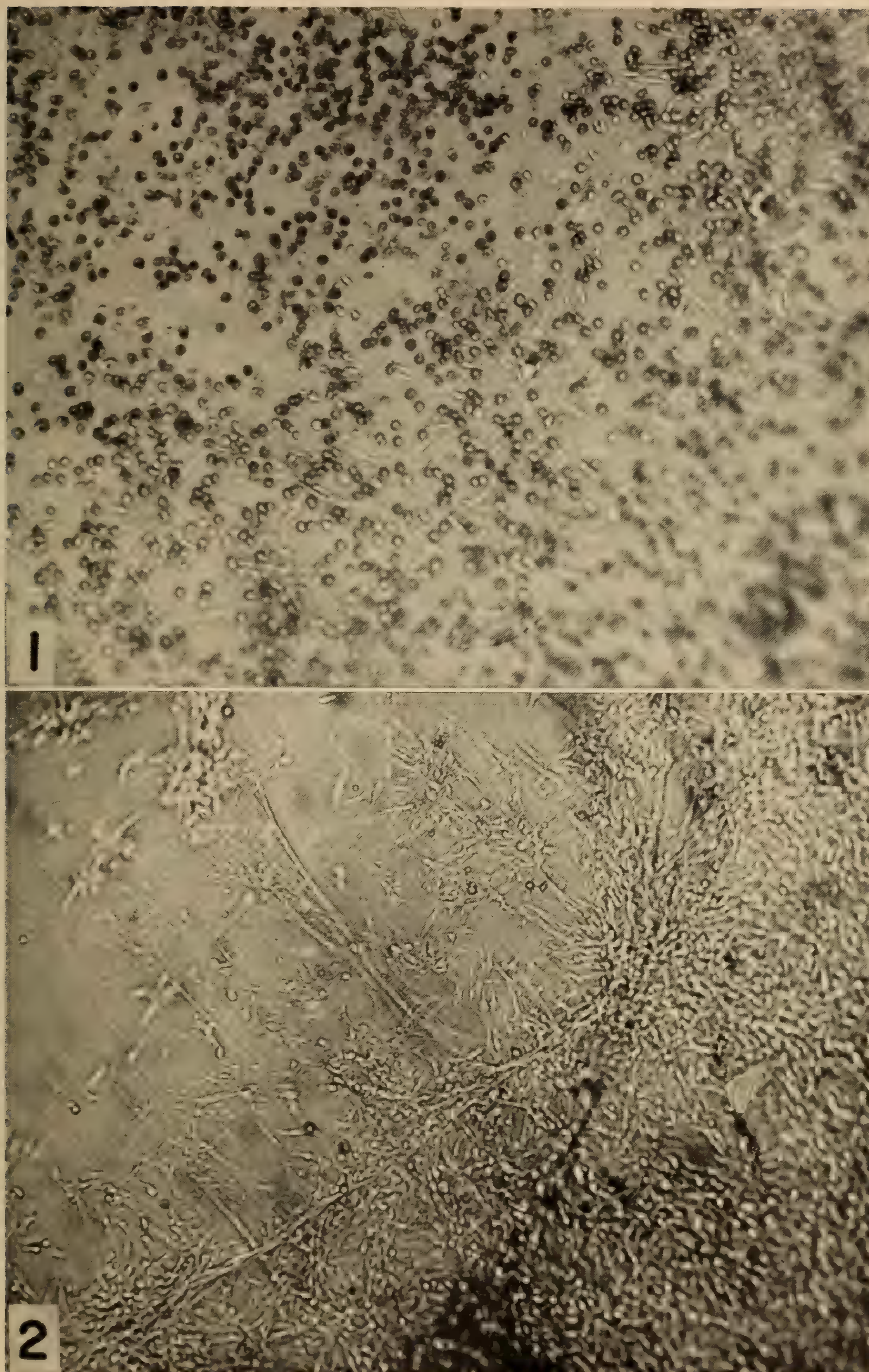
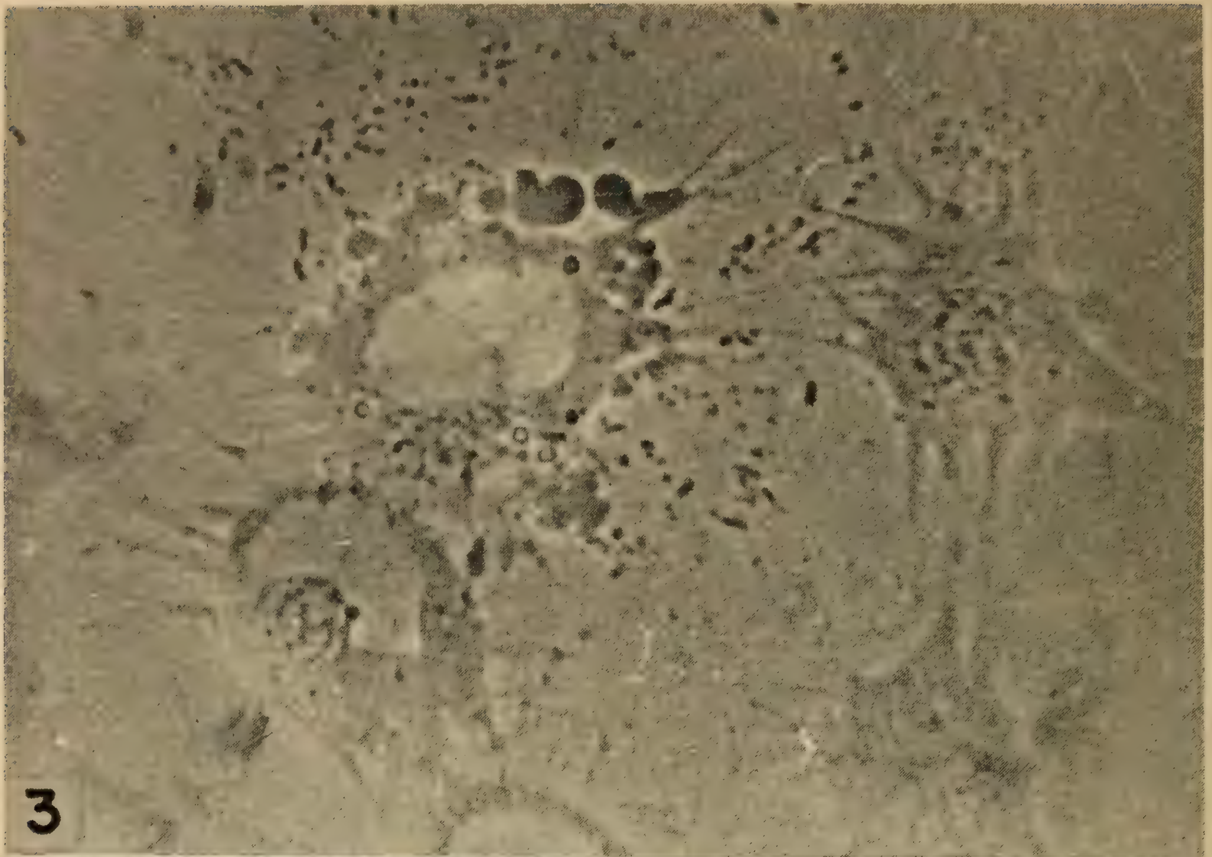
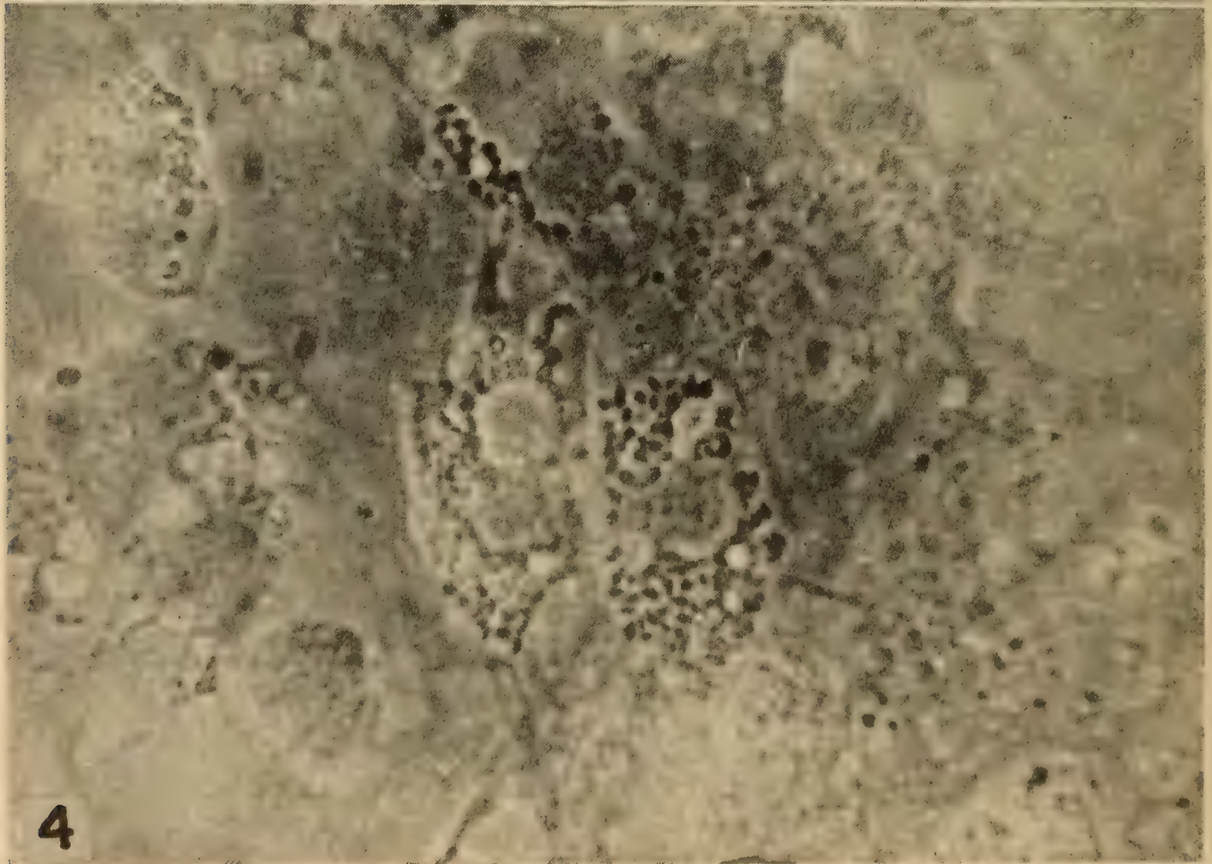


FIGURE 1. Amebocyte culture before addition of toxin. Note the discoid granular cells. Light areas represent some "hyaline," extended cells. (80 ×)

FIGURE 2. Same culture as in Figure 1, after addition of toxin. Amebocytes have lost granules and extended; many have apparently disintegrated.





FIGURES 3–4.

either degenerate or pathological, may be rod- or dumbbell-shaped and may be larger or smaller than the normal. *Translucent granules* are those which have lost their normal high refractility. They are usually a pale yellow-green.

Results

In vitro survival of amebocytes

Variation in serum concentration. In the hope that a lower concentration of Limulus serum frequently renewed might lead to cellular growth, four different concentrations were tested: 10, 25, 50 and 100 per cent, some with glucose added. The cells remained viable for as long as 36 days at the 10 and 50 per cent concentrations, but were not followed as long at the 25 and 100 per cent concentrations. Although long-term observations were not made with the 100 per cent (undiluted) serum in these first experiments, it was noted that granular discoid amebocytes were commonly found in the undiluted serum but rarely or never at the 10 per cent concentration. In all cases, the number of these cells gradually diminished. In general, cell appearance varies from the thin, flattened, hyaline cells with no granules and sharp, extended pseudopod-like processes, to the extended but granular cells and, finally, the granular discoid amebocyte. Attempts to transfer the amebocytes by direct explantation, trypsinization, or the use of "Versene" failed. In none of the direct microscopic examinations, nor in the stained preparations of the cultures were mitotic figures seen, nor was there any conversion of the amebocyte tissue into an organized growth.

Undiluted Limulus serum was therefore used in the tests with the bacterial toxin, and partially degranulated cells were consistently obtained. It was found unnecessary to replace the nutrient medium (the serum) at any time even in experiments of several weeks.

Effect of siliconized glassware. By applying silicone to the apparatus, in the very first experiment and in subsequent good cultures, the amebocytes were maintained morphologically indistinguishable from those in the animal for as long as 30 days in the rotating drum at room temperature without replenishment of the culture medium; more will be said about this in the section on interaction with bacteria.

Effects of the Limulus pathogen and its toxin on the blood cultures. Since the generalized pathology of the disease, an intravascular clotting, can be reproduced by a heat-stable toxin, the effects of this toxin on amebocytes *in vitro* were studied. As is shown in Table I, the addition of various dilutions of toxin to cultures of amebocytes in normal clean glassware caused prompt changes in morphology, culminating in the apparent disintegration of the cells, and the formation of an extracellular gel. The cells lost their granules, extended long processes, and assumed bizarre shapes. They are shown in Figures 1 and 2.

In order to study these changes under higher power, a loopful of fresh undiluted bacterial (*Vibrio*) suspension was mixed with a loopful of intact amebocytes from

FIGURE 3. Changes in amebocytes caused by *Vibrio*. Note blebs and needle-like extensions of cytoplasm, large vacuoles and nucleus displacement. (Phase, 1400 ×)

FIGURE 4. Amebocytes without addition of *Vibrio*. The two central cells are intact. Some vacuole formation is present in neighboring cells. (Phase, 1400 ×)

siliconized roller tubes and examined on a slide by phase microscopy at 1400 ×. Controls were (a) cells with the sterile artificial sea water without $NaHCO_3$, and (b) cells without such dilution.

A remarkable set of changes with the pathogen was observed. Destruction of the cell occurred within 5 minutes while the control cells, though definitely altered inasmuch as there was some pseudopod and vacuole formation, still contained

TABLE I

Effect of toxin on amebocytes in vitro

Dilution of toxin	Time following inoculation in hours		
	¼	1	5
Undiluted	+	++	+++
1×10^{-1}	+	++	+++
1×10^{-2}	+	++	++
1×10^{-3}	+	++	++
1×10^{-4}	0	0	0
Control (sterile artificial sea water without $NaHCO_3$)	0	0	0

0 = Majority of amebocytes discoid, granular.
+ = Small fraction of amebocytes extended and agranular ("hyaline").
++ = Larger fraction of amebocytes extended and agranular; some disintegrated.
+++ = Majority of amebocytes extended and agranular or disintegrated.

normal granules and were not greatly changed. The changes in the cells with the *Vibrio* were:

(1) A rapid and uncoordinated protrusion and retraction of blebs of cytoplasm, reminiscent of pseudopod formation.

(2) The formation of multiple intracytoplasmic vacuoles and rapid loss of granules from the cell. The few granules which remained in the cell seemed enlarged and lost their normal refractility; later the extruded granules also enlarged and became translucent.

(3) Coalescence of the vacuoles into larger clear ones which occupied most of the cell and which often displaced the nucleus. Formation of sharp, needle-like extensions of the cytoplasm.

(4) Marked ballooning of the cell and cytolysis.

Figures 3 and 4 depict some of these changes.

The addition of the Alkaligenes-like bacteria produced no comparable changes in intact amebocytes during the same period of time, although here, too, some pseudopod and vacuole formation were observed.

An extracellular gel is present both during the normal clotting process and the pathological intravascular clotting (Bang, 1956). When the pathogen was added to the cultures, both siliconized and normal, a much heavier gel-like material invariably appeared in the medium and persisted as long as the cultures were observed. Microscopic examination showed no living cells, but a ropy network which enmeshed numbers of agglutinated swollen, polymorphic, translucent, spher-

ical or elongated bodies which probably were altered granules. Small discrete dark bodies were also frequently found with these.

Antibacterial action of the discoid granular cells. When the cultures of cells on siliconized glass were first set up no sterile precautions were taken, and yet the cultures remained free of bacteria. It was therefore of immediate interest to test the ability of these "intact" cells to eliminate bacteria. It was found that large numbers of the Alkaligenes-like bacterium were rapidly eliminated by these cells, whereas cultures in which the cells had changed to the extended agranular type (unsiliconized glassware) showed reduction and inhibition of the bacteria but that this was temporary. Tables II and III summarize typical experiments.

TABLE II

Effect of granular discoid amebocytes on Alkaligenes-like bacterium. Number of colonies recovered per loopful of fluid and tissue

Number of bacteria added*	Hours after addition of bacteria							
	1¼	6	12	24	48	72	96	120
2.4×10^7	3 12	0 0	0 0	0 0	0 0 0	0 0 0	0 0 0	0 0 0
2.4×10^5	11 12	0 0	0 0	0 0	0 0 0	0 0 0	0 0 0	0 0 0
2.4×10^3	0 0	0 0	0 0	0 0	0 0 0	0 0 0	0 0 0	0 0 0
Sterile artificial sea water without $NaHCO_3$	0	0	0	0	0	0	0	0
Nothing added	0	0	0	0	0	0	0	0

* Varying dilutions of a fresh suspension of bacteria were added to the cultures. The figures given were determined by the pour-plate method. Three tubes were used for each dilution but samples were withdrawn from only two up to 24 hours. This served as a check on a possible influence of the sampling procedure itself.

The apparent drop in bacterial count at 1¼ hours might be explained by an estimated dilution of about 10^{-2} to 10^{-3} in the taking of small samples. However, at 6 hours tubes of the intact system showed no bacteria and from then on none were recovered from the granular cells. In cultures of extended cells (Table III) there seemed to be some inhibition but between the 24- to 72-hour sampling the bacterium reappeared and, by 72 hours, confluent growth was obtained. At the highest dilution (8.5×10^1 bacteria) no organisms were recovered at any time.

The experimental and control cultures were observed microscopically and loopfuls of fluid and tissue were examined on slides at 3½, 24 and 120 hours. There was no persistent gelation in the uninfected system or in those cultures which overcame the infection. The medium in these remained transparent and blue.

TABLE III

Effect of agranular extended amebocytes on Alkaligenes-like bacterium. Number of colonies recovered per loopful of fluid and tissue

Number of bacteria added*	Hours after addition of bacteria					
	2	6	12	24	48	72
8.5×10^5	42 33	4 9	0 1	1 0	80 Confluent 75	Confluent Confluent Confluent
8.5×10^3	10 5	1 0	0 0	0 1	50 100 3	Confluent Confluent Confluent
8.5×10^1	0 0	0 0	0 0	0 0	0 0 0	0 0 0
Sterile artificial sea water without $NaHCO_3$	0	0	0	0	0	0
Nothing added	0	0	0	0	0	0

* See footnote to Table II.

Cell changes were proportional to the number of bacteria inoculated. Cultures inoculated with 2.4×10^7 bacteria showed a majority of degranulated and flattened (but living) cells; those with 2.4×10^5 organisms had approximately equal proportions of degranulated and intact amebocytes, whilst the 2.4×10^3 tubes showed a great majority of intact cells, as did both controls. Furthermore, all cultures had apparently normal, free granules in the medium throughout the period of observation, with the greatest numbers at the highest and fewest at the lowest bacterial inoculum size and in the controls. Agglutinated clumps of dead bacteria were found in the experimental tubes.

The granular discoid cell cultures were ineffective in preventing the multiplication of the pathogenic *Vibrio*. Cultures inoculated with 10, 100, 1000 and 10,000 viable bacteria (direct colony count) showed destructive progressive infection, all

TABLE IV

Range of antibacterial activity of intact system against various unidentified marine bacteria

Bacterium stock number	Antibacterial activity
4 10 15 (slow grower) 19	Good
14 18 (slow grower)	Intermediate
12 17	None

TABLE V

Degenerative changes in amebocyte granules in serum (room temperature) (unsiliconized glassware)

Time (hours) elapsed subsequent to explantation of blood					
$\frac{1}{4}$	$\frac{1}{2}$	$4\frac{1}{2}$	$17\frac{1}{2}$	43	72
Few free in serum; all normal in shape, size, color and Brownian movement	Several in serum; all normal in shape, size, color and Brownian movement	Many in serum; all normal in shape, size, color and Brownian movement	Profuse in serum; most normal, some polymorphic, translucent, with altered Brownian movement	Profuse in serum; most polymorphic, translucent, some with red tinge and altered Brownian movement	Profuse in serum; all polymorphic, most with red tinge and altered Brownian movement

yielding confluent growth of bacteria 24 hours after inoculation. This experiment was repeated with the same result several times.

The effect of these "intact" siliconized cultures was then studied on several unidentified marine bacteria (Table IV). The activity was considered good if 1000 or more bacteria were eliminated. Thus the antibacterial activity was not limited to one strain of bacterium.

Eight different bacteria were used and three dilutions per bacterium, as described for the Limulus nonpathogen. The activity was studied at 2, 34 and 144 hours at room temperature.

An attempt was made to determine which of the components within the blood tissue culture system produced antibacterial activity. First, using unsiliconized glassware, the degeneration time of the cytoplasmic granules, once they had left the cell, was determined. Then antibacterial measurements were made with sera containing minimal and maximal numbers of granules as well as with whole blood homogenates at room temperature and at 0° C.

TABLE VI

*Antibacterial activity of whole blood homogenate on Alkaligenes-like bacterium at 0° C**

	Number of bacteria recovered per loopful		
	Hours after addition of bacteria		
	2	24	48
Fresh homogenate + 1×10^7 bacteria	Confluent	102‡	100
Control† + 1×10^7 bacteria	Confluent	Confluent	Confluent
Fresh homogenate + 1×10^5 bacteria	Confluent	16	12
Control + 1×10^5 bacteria	Confluent	Confluent	Confluent
Fresh homogenate + 1×10^3 bacteria	Confluent	9	7
Control + 1×10^3 bacteria	Confluent	50	Confluent

* After 48 hours at 0° C. all test and control tubes were returned to room temperature and so maintained for another 24 hours, when loopfuls were plated. Confluent growth was obtained in all cases.

† Control = 2 ml. of whole blood homogenate heated at 60° C. for $\frac{1}{2}$ hour.

‡ These figures represent the average of two experimental culture tubes.

Table V shows the degeneration time of the cytoplasmic granules outside of the cells to be between 17½ and 43 hours subsequent to explantation. This is interesting because of possible correlation with the 24- to 48-hour inhibition of bacterial growth seen in the extended cell system (Table III) and also because the discoid granular cell culture shows many perfectly normal granules 120 hours after addition of large numbers of the nonpathogenic Bacterium #5.

In tests for antibacterial activity of sera containing varying numbers of granules and of whole blood homogenates, equivocal results were obtained until the test for inactivation of the bacteria by a homogenate was done at 0° C. This has been found favorable for the measurement of the antibacterial activity of *Phascolosoma gouldii* blood (Bang and Krassner, 1958).

Table VI presents the results of this preliminary experiment.

Discussion

The *in vitro* maintenance of Limulus amebocytes was described by Loeb (1920), who reported that hanging-drop cultures made from his "experimental amoebocyte tissue" contained well preserved cells showing ameboid movements for over a week, but eventually showed "degenerative changes." Cultures showed active amebocytic migrations but no multiplication. As early as 1905 and 1906, the same worker had observed the preserving effects, on the cells, of solutions of both dilute acid and alkali in isotonic NaCl (Loeb, 1905, 1907, 1910) and had found (Loeb, 1907, 1910, 1928) that the irreversible conversion of discoid, granular amebocytes to an extended, agranular, "hyalinized" state upon exposure to glass could be delayed by coating the surface with Vaseline or paraffin. The later publications of Loeb and associates (Loeb and Blanchard, 1922; Loeb, Bierman and Gilman, 1924; Loeb, Bierman and Genther, 1925; Loeb and Genther, 1927; Loeb and Genther, 1928) dealt primarily with the stimulating effect of acid and alkali on the cell migrations ("outgrowths").

It is believed that the 30-day maintenance period for amebocytes in an intact state in a siliconized system, as reported in the present paper, is the longest recorded, as is the 36-day period of viability for amebocytes in an extended hyaline state. Such prolonged maintenance of cells at 20°–25° C. without change of medium is in itself interesting. Our results are in agreement with those of Loeb in that (a) undiluted Limulus serum is the best natural medium for amebocytes (Loeb, 1920) and (b) that no cell multiplication occurs in such cultures (Loeb, 1920; Loeb, Bierman and Genther, 1925).

The siliconized system seems to be well suited for conducting the various experiments described. The observations regarding *in vitro* destructive effects of the toxin on the cells, progressive infection of the cultures, and cell changes produced by the pathogenic *Vibrio* itself *in vitro*, correlate well with effects previously noted (Bang, 1956) in the living animal, thus providing some indication as to the probable course of events in pathogenesis and strongly implicating the toxin in the production of pathology following inoculation of the organism. It should be noted here that Métalnikov (1927) demonstrated that injection of endotoxins of several different bacteria (one, even after boiling at 100° C.) were lethal to caterpillars of *Galleria melonella*.

The suggestion has been made (Bang, 1956) that amebocyte reactions to trauma

and infection are ancient and represent basic protective mechanisms. Gelation of the blood would serve to immobilize the bacteria. During the course of several experiments conducted in this investigation it was noted that, while the pathogenic *Vibrio* was motile in the siliconized cultures, the nonpathogen was rapidly agglutinated and killed. Thus, trauma could stimulate interactions between cells and fluid resulting in a clot preventing further ingress of bacteria, those already within being killed by defensive factors in the blood. The results reported in the present paper indicate that defense mechanisms against hemorrhage (gelation, clotting) and against infection (production of antibacterial substance or substances) are intimately linked in the interaction between cellular and fluid elements of Limulus blood. Loeb, as noted elsewhere (Bang, 1956), described two stages in clotting and pointed out (1902) that fibers of clotted blood are partly formed directly from amebocyte protoplasm. These observations were confirmed during the course of this work. The formation of an extracellular gel during the normal clotting process, during pathological intravascular clotting and following addition of the pathogenic *Vibrio* or its toxin to siliconized or unsiliconized cultures, offers further evidence regarding the intimacy between the protective mechanisms. The *in vitro* gel persisted after addition of the pathogen and contained pathologically altered intra- and extracellular granules in large numbers. This suggests that the granules may be involved in some way, and is supported by the finding that they (a) appear normal in the media of granular cultures which have eliminated the Alkaligenes-like bacteria, their numbers increasing with increased bacterial inoculum size; (b) appear altered not only in cultures infected with the pathogen but also in blood from infected animals (Bang, 1956); and (c) have a degeneration time, in serum at 20–25° C., of 17½–43 hours (Table V) which may correlate well with the 24–48 hour antibacterial activity seen in an extended cell system (Table III). Finally, pretreatment (parenteral) of Limulus with the *Vibrio* toxin renders the animal more susceptible to infection with the *Vibrio* as well as with certain nonpathogenic bacteria (unpublished observation), this being presumably due to a lack produced by the toxin, of normal amebocytes and, possibly, of granules.

It has been shown that the intact system alone can eliminate large numbers of marine bacteria of *more than one strain* (Table IV), which lends support to the thesis that the phenomenon is truly antibacterial and not simply a chance observation. The extended cell and whole blood homogenate systems merely exhibit different levels of temporary antibacterial activity. An explanation may be that the intact system provides a steady, pump-like activity on the part of the healthy granular amebocytes leading to the release of some thermolabile intracellular component(s) into the serum long enough to kill all of the bacteria. The fact that as few as 10–100 pathogenic organisms can multiply and overcome the system may well be ascribed to the inactivating action of their toxin on the hypothetical intracellular component(s) and, possibly, on the granules. Its destructive action on the amebocyte itself has already been noted.

Summary

1. For effective *in vitro* study of the defense mechanisms against bacterial infection of *Limulus polyphemus* an unsiliconized and a siliconized culture system for blood cell maintenance were developed. The former contained viable but

partially degranulated amebocytes for at least 36 days, whilst the latter system kept the cells in an intact state resembling the *in vivo* for up to 30 days. Replenishment of the culture medium (undiluted Limulus serum) and use of antibiotics were found unnecessary. No cell multiplication was noted.

2. The reactions of the blood elements to certain nonpathogenic and pathogenic marine bacteria (and to their toxin) were studied. When a pathogenic *Vibrio* was added to the siliconized system of granular, discoid cells, persistent gelation of the medium regularly accompanied bacterial growth, the gel containing pathologically altered amebocytes and granules. While gelation is characteristic for shed normal Limulus blood, intact cultures—both uninfected as well as those which overcome the nonpathogenic Alkaligenes-like bacterium—often showed either absence or ephemeral presence of gel in the medium. Such cultures also contained normal granules and showed large clumps of agglutinated dead bacteria consistently, suggesting involvement of the gelation process in immobilization and, perhaps, killing of the invading organism. The culture systems were active against several strains of marine bacteria. Experiments with a nonpathogenic Alkaligenes-like bacterium showed that only the intact, granular cultures showed rapid (6 hours at 20–25° C.) and *permanent* elimination of large numbers (inoculum sizes up to several million), the unsiliconized extended cell and whole blood homogenate systems merely exhibiting different levels of evanescent activity.

3. The successful infection of the intact system, by very small numbers (10–100), of pathogenic *Vibrio* clearly established its *in vitro* pathogenicity. Its thermostable toxin caused prompt changes in the morphology of the intact amebocytes in culture, even at a 10^{-3} dilution, and a phase microscopic study of the action of the *Vibrio* itself on the cells revealed rapid cellular alterations culminating in cytolysis.

LITERATURE CITED

Bang, F. B., 1956. A bacterial disease of *Limulus polyphemus*. *Bull. Johns Hopkins Hosp.*, **98**: 325–351.

Bang, F. B., and S. M. Krassner, 1958. Antibacterial activity of *Phascolosoma gouldii* blood. *Biol. Bull.*, **115**: 343.

Lankester, E. R., 1884. On the skeleto-trophic tissues and coxal glands of Limulus, Scorpio and Mygale. *Quart. J. Micr. Sci.*, **24**: 129–162.

Loeb, L., 1910. Über die Blutgerinnung bei Wirbellosen. *Biochem. Zeitschr.*, **24**: 478–495.

Loeb, L., 1902. On the blood lymph cells and inflammatory processes of Limulus. *J. Med. Res.*, **7**: 145–158.

Loeb, L., 1920. The movements of the amoebocytes and the experimental production of amoebocyte (cell-fibrin) tissue. *Washington Univ. Studies*, **8**: 3–79.

Loeb, L., 1905. Studies on cell granula and amoeboid movements by the blood cells of Limulus. *Univ. Pennsylvania Med. Bull.*, **18**: 91–95.

Loeb, L., 1907. Untersuchungen über die Granula der Amoebozyten. *Folia haemat.*, **4**: 313–322.

Loeb, L., 1910. Über den Einfluss von chemischen und physicalischen Umgebungsänderungen auf die Blutzellen von Limulus, und insbesondere auf ihre Granula. *Pflüger's Arch.*, **131**: 465–508.

Loeb, L., 1928. Amoebocyte tissue and amoeboid movement. *Protoplasma*, **4**: 596–625.

Loeb, L., and K. C. Blanchard, 1922. The effect of various salts on the outgrowth from experimental amoebocyte tissue near the isoelectric point and with the addition of acid and alkali. *Amer. J. Physiol.*, **60**: 277–307.

Loeb, L., H. Bierman and I. P. Genther, 1925. The effect of various ions on the experimental amoebocyte tissue of Limulus and their interaction with other variable factors. *Arch. exper. Zellforschg.*, **1**: 17–288.

LOEB, L., J. M. BIERMAN AND E. GILMAN, 1924. The effect of acid on the amoebocyte tissue of Limulus in tissue cultures. *Proc. Soc. Exp. Biol. Med.,* **21**: 245–248.

LOEB, L., AND I. T. GENTHER, 1927. The effect of alkali on amoebocyte tissue of Limulus. *Arch. exper. Zellforschg.,* **5**: 83–110.

LOEB, L., AND I. T. GENTHER, 1928. The effect of acid on amoebocyte tissue of Limulus. *Arch. exper. Zellforschg.,* **5**: 355–378.

MÉTALNIKOV, S., 1927. L'infection microbienne et l'immunité chez la mite des abeilles *Galleria melonella.* Monographies de l'Institut Pasteur, Masson et Cie, Éditeurs, Paris.

SHIRODKAR, M. V., F. B. BANG AND A. WARWICK, 1958. Antibacterial action of Limulus blood in an *in vitro* system. *Biol. Bull.,* **115**: 341.

WARWICK, A., AND F. B. BANG, 1957. Survival of marine invertebrate cells in tissue culture (Limulus and Ostrea). *Biol. Bull.,* **113**: 334.